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Role for ETS domain transcription factors Pea3/Erm in mouse lung development

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Abstract

During the development of the mouse lung, the expression of a number of genes, including those encoding growth factors and components of their downstream signaling pathways, is enriched in the epithelium and/or mesenchyme of the distal buds. In this location, they regulate processes such as cell proliferation, branching morphogenesis, and the differentiation of specialized cell types. Here, we report that the expression of *Pea3* and *Erm* (or *Etv5, Ets variant gene 5*), which encode Pea3 subfamily ETS domain transcription factors, is initially restricted to the distal buds of the developing mouse lung. *Erm* is transcribed exclusively in the epithelium, while *Pea3* is expressed in both epithelium and mesenchyme. *Erm/Pea3* are downstream of FGF signaling from the mesenchyme, but their responses toward different FGFs are not the same. The functions of the two proteins were investigated by transgenic expression of a repressor form of Erm specifically in the embryonic lung epithelium. When examined at E18.5, the distal epithelium of transgenic lungs is composed predominantly of immature type II cells, while no mature type I cells are observed. In contrast, the differentiation of proximal epithelial cells, including ciliated cells and Clara cells, appears to be unaffected. A model is proposed for the role of Pea3/Erm during the dynamic process of lung bud outgrowth and proximal–distal differentiation, in response to FGF signaling. Our results provide the first functional evidence that Pea3 subfamily members play a role in epithelial–mesenchymal interactions during lung organogenesis.

Keywords: Lung; FGF; Erm; Pea3; ETS domain proteins

Introduction

Mouse lung development starts from E9.5 when two primordial buds appear in the ventral foregut just anterior to the stomach. It can be histologically divided into four stages: (1) the pseudoglandular stage (E9.5–E16.5), when the bronchial and respiratory tree is formed from the primordial buds and is lined with epithelial precursor cells, (2) the canalicular stage (E16.5–E17.4), (3) the terminal sac stage (E17.4 to P5), and (4) the alveolar stage (P5–P30). During the later stages, the precursor cells differentiate into various cell types lining the proximal and distal airways, followed by the formation of alveoli and a mature vascular system (reviewed in Warburton et al., 2000).

Between E9.5 and E16.5, the primary buds undergo repeating rounds of outgrowth and branching, leading to a tree-like structure. Coincident with morphological changes, the lung buds also undergo proximal–distal patterning. In the case of the epithelium, this leads to the differentiation of the unspecialized, multipotent endodermal precursor cells into the mature cell types characteristic of the proximal and distal airways (Warburton et al., 2000). In vivo cell lineage studies indicate that the epithelial precursors for the trachea and large bronchi versus more peripheral airways are segregated at a relatively early stage (before E11.5) (Perl et al., 2002). However, transplantation experiments demonstrate that the epithelial precursor cells are able to differentiate along alternative pathways in response to diffusible molecules produced by the mesenchyme from different parts of

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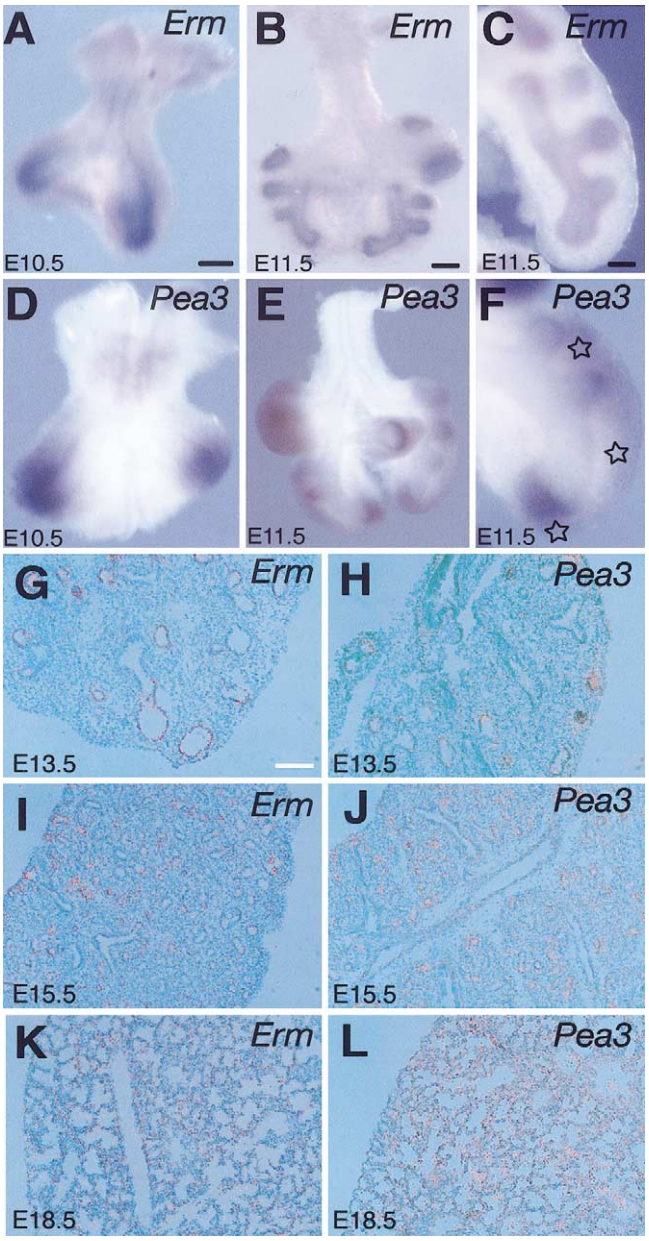


Fig. 1. Spatial and temporal expression patterns of *Erm* and *Pea3* during lung development. (A–F) Whole mount and (G–L) radioactive section in situ hybridization. *Erm* is expressed in the distal lung endoderm at E10.5 (A), and continues at E11.5 (B, C), E13.5 (G), E15.5 (I), and E18.5 (K). *Pea3* is expressed in distal lung endoderm and mesoderm at E10.5 (D), and continues at E11.5 (E, F), E13.5 (H), E15.5 (J), and E18.5 (L). (C, F) *Erm* is expressed in distal endoderm (C), while *Pea3* is expressed in distal endoderm and mesoderm (star) (F). Scale bar, 100 μm for (A) and (D) 150 μm for (B) and (E), 180 μm for (C) and (F), 100 μm for (G–L).

the embryonic lung (Wessells et al., 1970; Shannon et al., 1994, 1998). Further studies indicate that the processes of branching morphogenesis, proximal–distal patterning, and differentiation are regulated by reciprocal interactions between the epithelial cells, the surrounding mesenchyme, and the outermost mesothelium, mediated by extracellular signaling molecules, cell membrane–bound receptors, and intracellular signaling pathways (for review, see Hogan et al., 1997; Hogan and Yingling, 1998; Warburton et al., 2000; Cardoso, 2001).

From previous studies, it is known that members of several conserved families of signaling molecules, including the FGF, Hh, Bmp/TGFβ, Wnt, EGF, and PDGF families, are expressed specifically in the distal tips of the developing lung (see reviews cited above). These structures therefore behave as "signaling centers," modulating and driving the process of branching morphogenesis as well as proximal–distal differentiation. For example, *Fgf10* is expressed in the mesenchyme just adjacent to the bud tip and acts as a chemoattractant factor directing lung bud outgrowth (Bellusci et al., 1997; Min et al., 1998; Sekine et al., 1999; Lebeche et al., 1999; Weaver et al., 2000). Other *Fgfs* expressed in the embryonic lung include *Fgf1 Fgf2, Fgf7,*

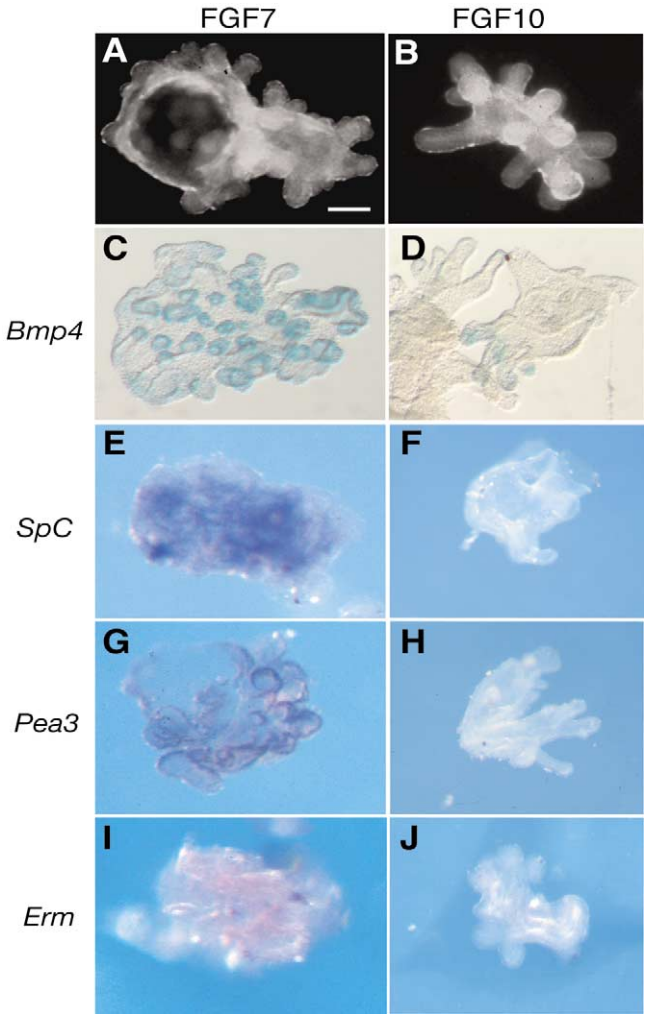


Fig. 2. Regulation of *Pea3/Erm* expression by FGF7 and FGF10 in mesenchyme-free endoderm culture. Distal endoderm buds from E11.5 lungs were cultured in Matrigel for 48 h with 20 ng/ml FGF7 (A, C, E, G, I) or 250 ng/ml FGF10 (B, D, F, H, J). After culture, the samples were released from the Matrigel by enzyme treatment and the expression of distal marker genes examined by lacZ staining for *Bmp4-lacZ* (C, D), or by in situ hybridization for *SpC* (E, F), *Pea3* (G, H), and *Erm* (I, J). Scale bar, 100 μm.

Fgf9, and *Fgf18* (Gonzalez et al., 1990; Fu et al., 1991; Han et al., 1992; Mason et al., 1994; Finch et al., 1995; Bellusci et al., 1997; Hu et al., 1998; Ohbayashi et al., 1998; Colvin et al., 2001; Hyatt et al., 2002). *Fgf7* is expressed in lung mesenchyme (Mason et al., 1994; Bellusci et al., 1997; Lebeche et al., 1999), and is thought to participate in the specification of distal epithelial cell types (Ulich et al., 1994; Shiratori et al., 1996; Cardoso et al., 1997; Shannon et al., 1999; Hyatt et al., 2002). *Fgf9* is initially expressed in the epithelium and mesothelium, and later is restricted to the mesothelium, where it is thought to be responsible for maintaining *Fgf10* expression and proliferation of the underlying mesodermal (Colvin et al., 2001).

Many ETS domain transcription factors are known to be downstream of FGF signaling (for review, see Sharrocks, 2001). Recently, we identified from a subtractive hybridization and differential screen two members of the Pea3 subfamily of ETS factors, *Pea3* and *Erm*, as being specifically expressed in distal, but not proximal, lung buds epithelium at E11.5 (Liu et al., 2002). This finding is consistent with previous reports on the expression of the two genes in lung (Chotteau-Lelievre et al., 1997; de Launoit et al., 1997). The Pea3 subfamily includes three members: Pea3, Erm (or *Etv5*, Ets variant gene 5), and Er81 (or Etv1, Ets variant gene 1). All three have a 72-amino-acid N-terminal transcription activation domain, an 85-amino-acid winged helix–turn–helix ETS DNA binding domain, and a short C-terminal domain. They recognize similar DNA sequences flanking the core GGAA/T binding sequence and act as transcriptional activators (Laget et al., 1995; de Launoit et al., 1997 Bojovic et al., 2001). The subfamily has been implicated in various cellular processes, including proliferation, differentiation, and tumorigenesis (Bartel et al., 2000; Maroulakou and Bowe, 2000; Sharrocks, 2001). For example, members are overexpressed in oncogene-induced mouse mammary tumors, and expression of an inhibitor form of Pea3 reduces the size and number of tumors (Shepherd et al., 2001). In the nervous system, studies have shown that *Pea3* and *Er81* are differentially expressed in motor neuron pools in the chick spinal cord, as well as in subsets of muscle sensory neurons. The motor and sensory neurons innervating the same muscle express the same *Pea3* member, and this expression pattern is required for the formation of functional circuits (Lin et al., 1998; Arber et al., 2000; Price et al., 2002). During organogenesis, *Pea3* genes are differentially expressed in the mesenchyme and epithelium of the developing lung, salivary gland, gut, olfactory and otic organs (Chotteau-Lelievre et al., 1997). While *Erm* and *Pea3* are primarily expressed in the developing epithelium, *Er81* is mostly confined to the surrounding mesenchyme (Chotteau-Lelievre et al, 1997). Their expression patterns suggest that they participate in epithelial–mesenchymal interactions, but their specific roles in organogenesis have not been investigated previously. All three members have been genetically inactivated in mice. *Erm* null mutants die at E9.5, before the onset of the mor-

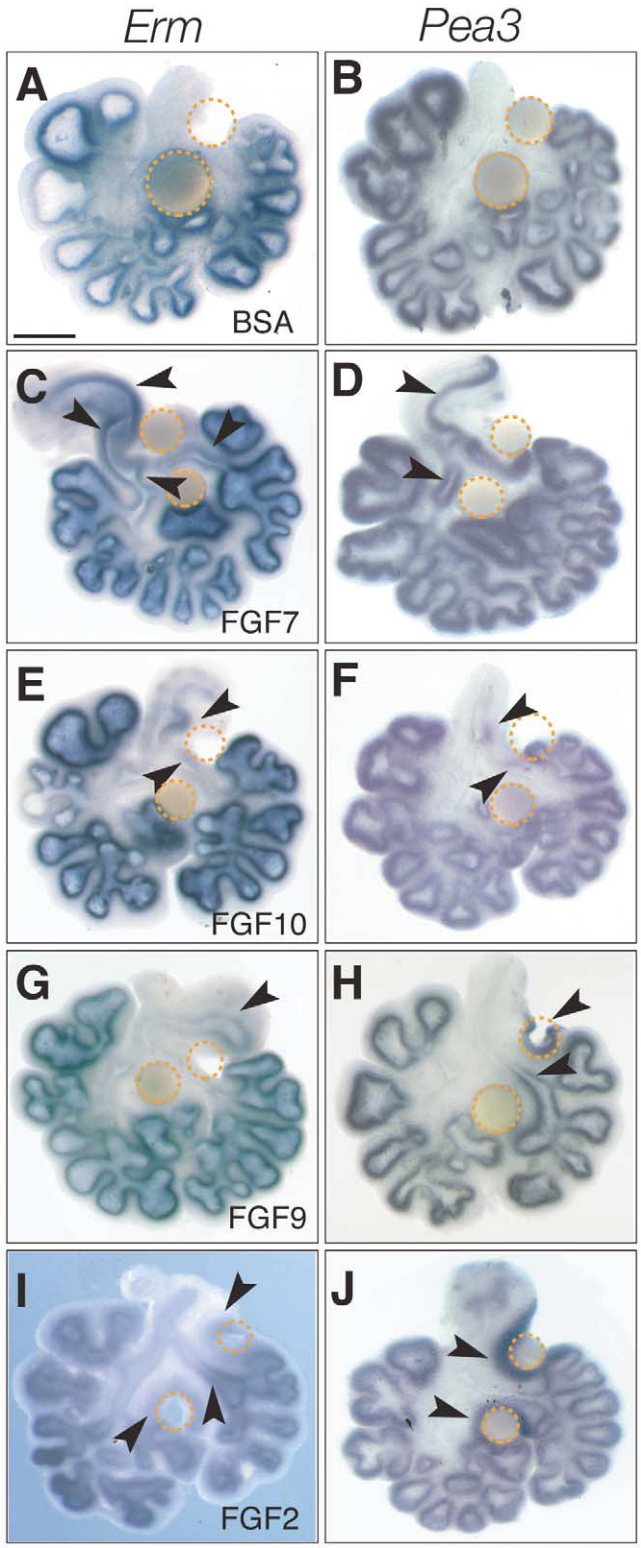


Fig. 3. Expression of *Erm* and *Pea3* is regulated by FGF signaling in the intact lung. FGF or BSA-soaked beads were implanted in the mesoderm of E11.5 lungs. After 24–48 h, gene expression was assayed by whole-mount in situ hybridization. (A, C, E, G, I) *Erm* expression. (B, D, F, H, J) *Pea3* expression. Beads were saturated with BSA (A, B); FGF7 (C, D); FGF10 (E, F); FGF9 (G, H); and FGF2 (I, J). Arrowheads indicate ectopic expression of *Erm/Pea3*. Yellow circles indicate the position of the implanted beads. Scale bar, 400 μm.

phogenetic events involved in lung and other organ development (T. Jessel, personal communication). *Pea3*−/− and *Er81*−/− mice both survive after birth, and apparently do not have major organogenesis defects (Arber et al., 2000; Laing et al., 2000), suggesting that Pea3 members have redundant functions during organ development.

Here, we study the function of Pea3/Erm during lung development by expressing a dominant repressor form of *Erm* specifically in the epithelium. Our results are consistent with the idea that Erm/Pea3 are downstream targets of FGF signaling from the mesoderm and function to promote or maintain the phenotype of distal epithelial cells.

Materials and methods

Lung explant culture

Embryonic lungs of ICR mice (Harlan-Sprague-Dawley, Indianapolis) were dissected at E11.5. For mesenchyme-free endoderm culture, distal endoderm was isolated after treatment with pancreatin/trypsin solution and cultured in growth factor reduced Matrigel in medium supplemented with 20 ng/ml FGF7 or 250 ng/ml FGF10 (Bellusci et al., 1997). Bud length was determined from 10 samples under each condition. Endoderm was harvested after 48 h by treatment with Matrisperse enzyme (BD Bioscience; Hyatt et al., 2002), fixed with 4% paraformaldehyde in PBS, and processed for in situ hybridization or β-galactosidase staining.

Intact E11.5 lungs were cultured for 24–48 h at 37°C in 5% CO_2 on Nuclepore polycarbonate filters (Whatman) floating on 500 μl medium (50% DMEM: 50% Ham's F12, 0.1% BSA, 0.05 U/ml penicillin, 0.05 μg/ml streptomycin). FGF- and BSA-soaked beads, prepared as described (Weaver et al., 2000), were implanted in the mesenchyme adjacent to proximal endoderm. FGFs were obtained from R & D Systems. At least two lungs were examined for each condition and the experiments repeated at least twice.

Luciferase reporter assay

Each well of a 24-well plate was seeded with 1.5×10^6 HEK293 cells or 1.0×10^6 Immorto mouse C3 cells. The latter epithelial cell line was derived from adult lungs of the transgenic H-2Kb-tsA58 (Immorto) mouse strain (Jat et al., 1991) by Dr. Robert Whitehead (Vanderbilt Medical Center, Nashville, TN) and was grown in the presence of 2.5 U/ml IFN-γ (Calbiochem). Approximately 20 h later, the cells in each well were transfected with 80 ng of a Pea3-responsive luciferase reporter containing 5 reiterations of a functional ETS binding site (AACGAGGAAGTATTA) cloned upstream of the minimal thymidine kinase promoter of the plasmid TK-Luc. This ETS site has been shown to be responsible for Pea3 subfamily transactivation of the matrilysin promotor (Crawford et al., 2001). Included in each transfection mix was 160 ng of either empty pCDNA3, pCS2-EngR, pFLAG-CMV-Erm290-489, or pFLAG-CMV-EngR-Erm290-489. For some experiments, the reporter was stimulated by including 80 ng of a CMV-promoter driven expression vector for wild type Erm. Total DNA was kept constant with empty pCDNA3 vector. Each transfection mix included 1 ng of CMV-RL reporter (Promega) to normalize for transfection efficiency. Cells were lysed 24 h later and assayed by using the Dual Luciferase Kit (Promega), according to the manufacture's directions. Each assay was performed in triplicate.

Generation of transgenic mice

A full-length mouse *Erm* cDNA (I.M.A.G.E. clone #3665646) was cloned into the *Eco*RI site of the human 3.7-kb SpC/SV40-small-T-intron/polyA vector. This regulatory element of *SpC* (*Sftpc*, here designated as *SpC*) specifically drives transgene expression in distal epithelium from E10.5, but high level of expression in these cells and in Type II cells is only achieved after E15.5 (Wert et al., 1993; and data not shown). For generating truncated Erm, only sequences corresponding to amino acids 290–489 were cloned into the vector. For the repressor form of Erm (EngR-Erm), the cDNA corresponding to amino acids 290 to 489 was inserted at the *Xho* and *Xba* sites into the pCS2-EngR plasmid containing the *Drosophila* Engrailed repressor domain (kindly provided by Dr. Gerald H. Thomsen). The Eng-Erm 290–489 fragment was then removed by digesting with *ClaI* and *Xba*I, blunt ended, and inserted into the SpC/SV40 vector, which had been linearized by *Sal*I and *Eco*RI. All PCR products were sequenced.

The expression cassette was excised with *Nde*I and *Not*I for the full-length and truncated Erm, and with *Nde*I and *Sma*I for the repressor form of Erm. Transgenic embryos were generated as described (Weaver et al., 1999) and identified by PCR using the following primers: 5′-AGGAACAAACAGGCTTCAAA-3′ (SpC promotor for 5′), and 5′-TGGCTACAGGACGACAACTCG-3′ (for full-length Erm), 5′-AGGAGGCCCCTCTCGATACAT-3′ (for the truncated Erm 290–489), and 5′-CACATCCACATCAATGTCGT-3′ (for EngR-Erm). For full-length and truncated Erm, two and five transgenic embryos were obtained, respectively. Nine SpC-EngR-Erm transgenic embryos were obtained at E18.5, and six of them showed an abnormal lung phenotype. One transgenic lung was harvested at E17.5 and showed the same abnormalities. No difference was seen in the overall size of the transgenic versus nontransgenic pups.

In situ hybridization

Whole-mount in situ hybridization was performed by using digoxygenin-labeled antisense riboprobes as described (Bellusci et al., 1997).

For section in situ hybridization, ^{35}S-labeled probes were prepared from the following templates: the 0.4-kb fragment

of small T antigen (Wert et al., 1993), 2.4-kb *Foxj1* (Hackett et al., 1995), 0.68-kb *SpC* (Wert et al., 1993), 0.454-kb rat *CC10* (*Scgblal*, also known as CCSP or uteroglobin) (Wert et al., 1993), and 0.35-kb mouse *Aquaporin-5* (Ramirez et al., 2000). Sections of transgenic and control lungs were processed at the same time. To quantitate the proportion of proximal airways in control and *SpC-EngR-Erm* transgenic lungs, four sections corresponding to similar regions of control and two transgenic lungs were examined. The ratio of proximal and distal airways was measured by comparing *Foxj1*-expressing area to the total area of the lung using NIH-image software. About 10% of the total area of control lungs and 25% of the total area of transgenic lungs express *Foxj1*.

Electron microscopy

Electron microscopy was performed as previously described (Weaver et al., 1999). Briefly, control and transgenic lung tissue was cut into 1- to 2-mm^3 pieces and fixed in 2% glutaraldehyde in phosphate buffer. After dehydration, the tissues were embedded in Spurr (Ted Pella, Redding, CA). Semithin sections were stained with ethylene blue and examined with light microscopy to select the field for ultrathin sectioning. Ultrathin sections were mounted on uncoated copper mesh grids, counterstained with uranyl acetate-lead, and examined using a Philips 300 electron microscope.

Antibody and β-galactosidase staining

Sections were dewaxed and rehydrated and, for antigen unmasking, heated to about 80°C in a microwave oven in 10 mM NaCitrate buffer (pH 6). After blocking in 5% normal donkey serum in PBS, sections were incubated overnight with primary antibody at 4°C and, after washing, with Cy3-conjugated secondary antibody. Nuclei were stained with 0.1 μg/ml DAPI for 5 min. The phosphohistone H3 antibody was from Upstate Technology and used at 1:500 dilution. The cleaved caspase-3 antibody was from Cell Signaling and was diluted to 1:50. Detection of pre-CC10 was kindly performed by Dr. Mildred Stahlman using antibody generously supplied by Dr. Jeffrey Whitsett (Whitsett et al., 2002).

β-Galactosidase staining was performed as described (Weaver et al., 2000).

Estimation of cell proliferation

To estimate epithelial cell proliferation, nuclei with positive phosphohistone H3 staining were scored in sections double stained with anti-phosphohistone H3 and DAPI. Distal and proximal airways were distinguished by morphology. Since squamous type I cells are difficult to distinguish from mesodermal cells in histological sections of E18.5 lungs, only nuclei immediately abutting the lumen of the sacs were scored as belonging to distal epithelial cells. For transgenic lungs, 43 phosphohistone positive nuclei were identified out of a total of 4173 nuclei. For control lungs, 10 phospho-histone positive nuclei were found out of 4923 counted. The mitotic index is calculated as the number of phosphohistone-positive nuclei per 100 nuclei counted. A similar method was used to compare the cell proliferation rate of proximal epithelial cells.

Results

Pea3 and Erm expression in the developing lung

From a subtractive hybridization and differential screen (Liu et al., 2002), *Erm* was identified as being specifically transcribed in distal, but not proximal, epithelium of the E11.5 mouse lung. Although transcription of *Erm* and other *Pea3* subfamily ETS genes has been detected previously in the embryonic lung (Chotteau-Lelievre et al., 1997; de Launoit et al., 1997), their precise spatial and temporal expression patterns in relation to branching morphogenesis have not been investigated. As illustrated in Fig. 1A–F, we find that both *Erm* and *Pea3* are expressed in the endoderm at the tips of the buds from the time they first emerge (E10.5), while *Pea3* is also expressed in the overlying mesoderm. Section in situ hybridization shows that expression of both *Erm* and *Pea3* continues up to at least E18.5 in cells of the distal airways (Fig. 1G–L).

Expression of Erm and Pea3 is positively regulated by FGFs

Pea3 proteins are posttranslationally modified in response to the MAPK and PKA signaling pathways (Janknecht et al., 1996), and in zebrafish *Erm* and *Pea3* are downstream of FGF signaling in the hindbrain (Raible and Brand, 2001; Roehl and Nusslein-Volhard, 2001). We therefore investigated the effect of FGF7 and FGF10 on *Erm* and *Pea3* expression in mesenchyme-free, distal lung epithelium cultured in Matrigel. In this culture system, the epithelium responds differently to these two FGFs. When 20 ng/ml FGF7 is added to the medium, the endoderm initially forms a cyst, which then develops numerous small buds (about 60 μm long) at the surface. In response to higher concentrations of FGF7 (250 ng/ml), the endoderm forms a large cyst without buds. In contrast, when 250 ng/ml FGF10, which is the lowest concentration to keep the endoderm healthy, is added to the culture, the endoderm gives rise to fewer, more elongated buds (about 150 μm long) and less cyst expansion (Fig. 2A and B; Bellusci et al., 1997). In addition, FGF7 and FGF10 have different effects on gene expression in this culture system. Our results indicate that *Bmp4* and *SpC* expression are induced to much higher levels by 20 ng/ml FGF7 than 250 ng/ml FGF10 (Fig. 2C–F), which is consistent with previous finding

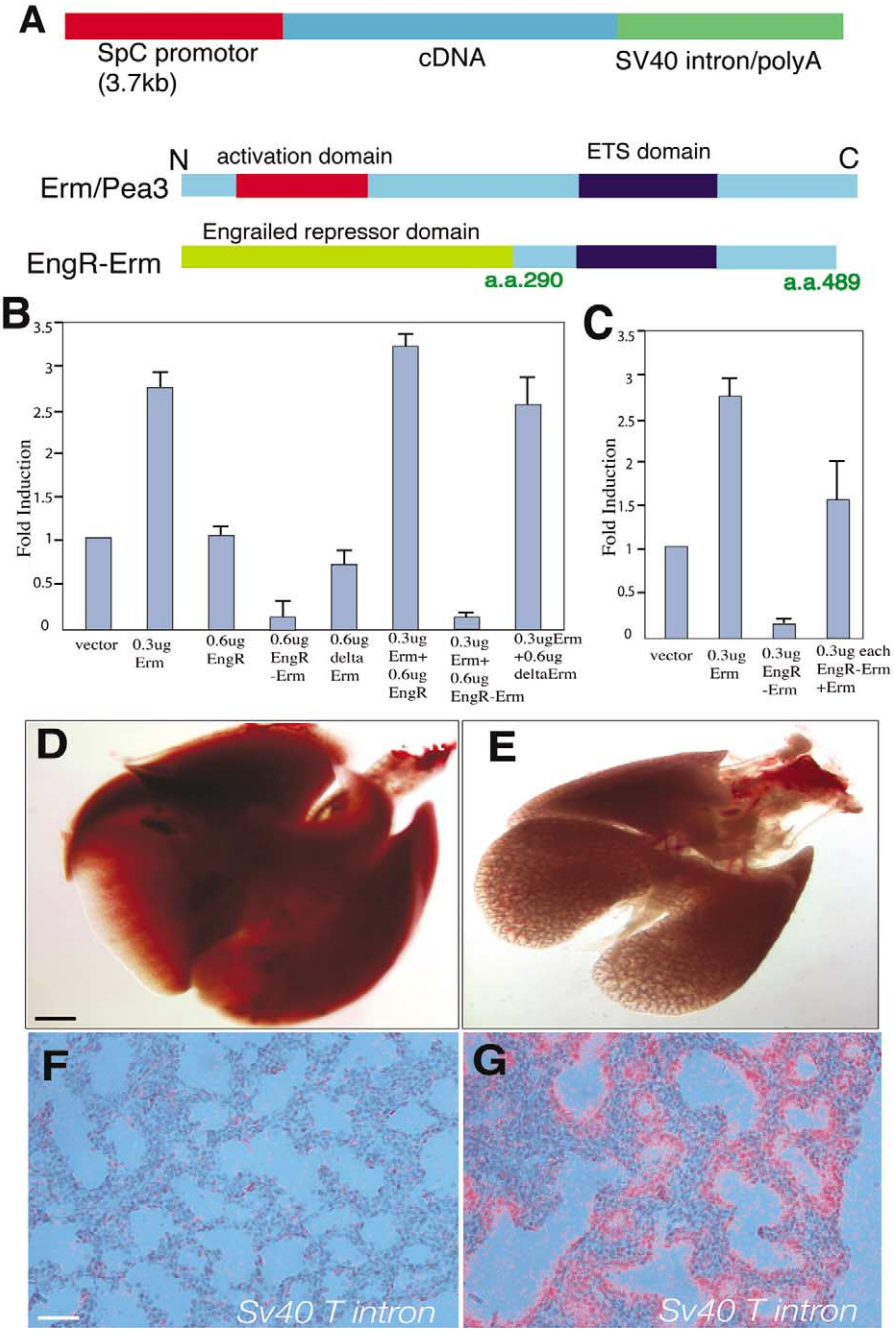
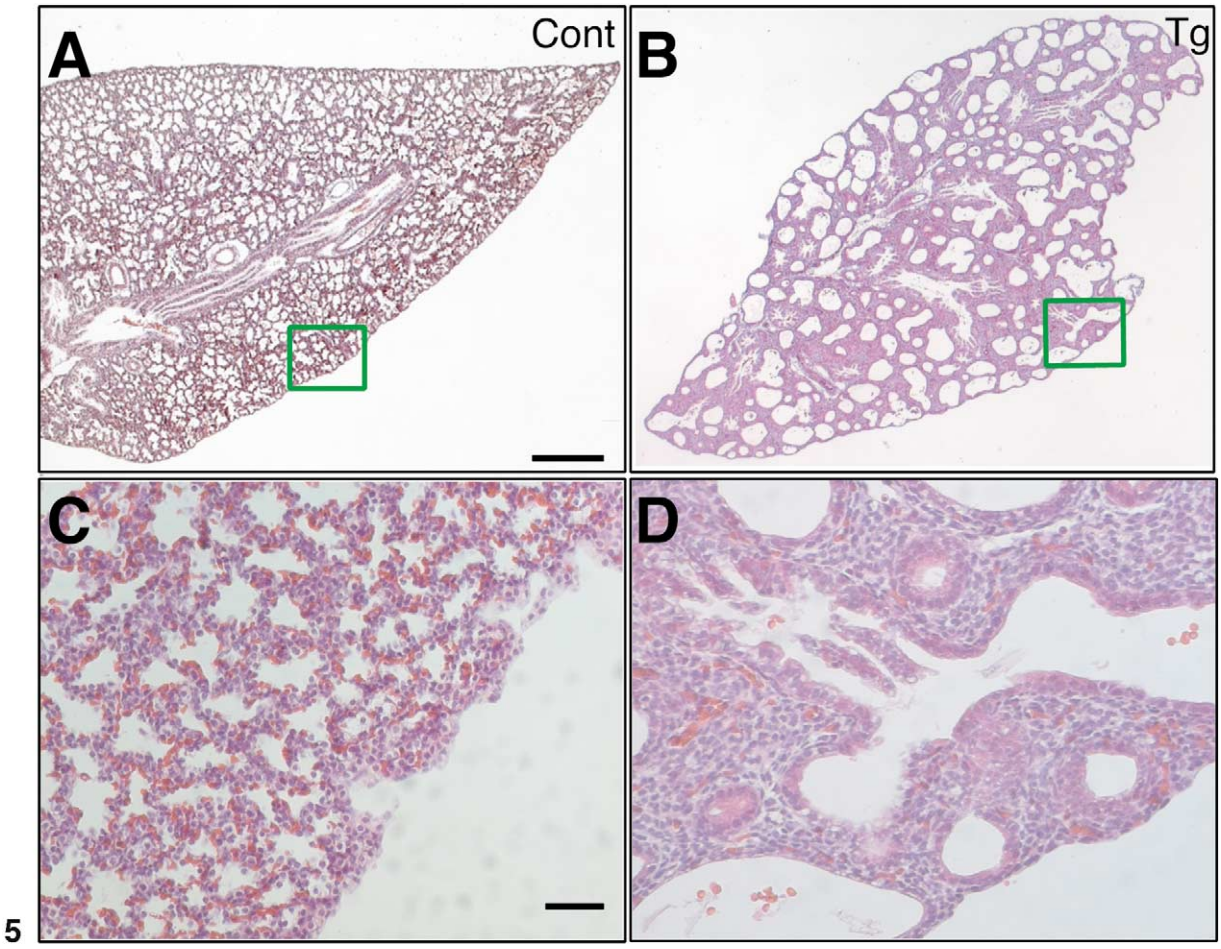


Fig. 4. Generating transgenic lungs expressing a repressor form of *Erm*. (A) The activation domain of Erm was replaced by the repressor domain of *Drosophila* Engrailed (a.a. 1-298). This engineered cDNA was subcloned into the transgene vector, downstream of the 3.7-kb human SpC promoter, and upstream of the *SV40* intron/polyA. (B, C) In vitro assays using a Pea3 responsive luciferase reporter (Crawford et al., 2001) show that, in both HEK 293 cells (B) and Immorto mouse C3 lung epithelial cells (C), full-length Erm acts as a transcriptional activator, while EngR-Erm acts as a repressor that effectively counteracts Erm activity. (B) In HEK 293 cells, wild type Erm acts as a transactivator and increases the expression of the luciferase reporter gene by more than 2.5-fold compared with vector alone control. While the Engrailed domain by itself (EngR) does not have any effect, EngR-Erm strongly represses the expression of the reporter gene, and delta Erm (truncated Erm 290-489) only slightly decreases the luciferase activity. When coexpressed with wild-type Erm, EngR by itself has little effect, while EngR-Erm very strongly, and delta Erm only slightly, represses Erm activity. (C) Similar results are obtained in Immorto mouse C3 lung epithelial cells. Wild-type Erm acts as a transactivator, EngR-Erm represses the expression of the reporter gene, and also effectively counteracts Erm when coexpressed with full-length wild-type protein. (D, E) Macroscopic phenotype of a typical E18.5 transgenic (E) compared with nontransgenic (D) lung. (F, G) Section in situ hybridization of tissue from the peripheral region of control (F) and transgenic (G) lung, using *SV40* T intron as probe. Scale bar, 1.5 mm for (D) and (E), and 50 μm for (F) and (G).

(Hyatt et al., 2002). Significantly, FGF7 gives stronger induction of *Pea3/Erm* expression than FGF10, and this effect is particularly striking for *Pea3* (Fig. 2G–J).

To see if this differential response is a feature of the Matrigel culture system or is also seen in intact lung tissue, we used a whole lung organ culture system in which FGF-



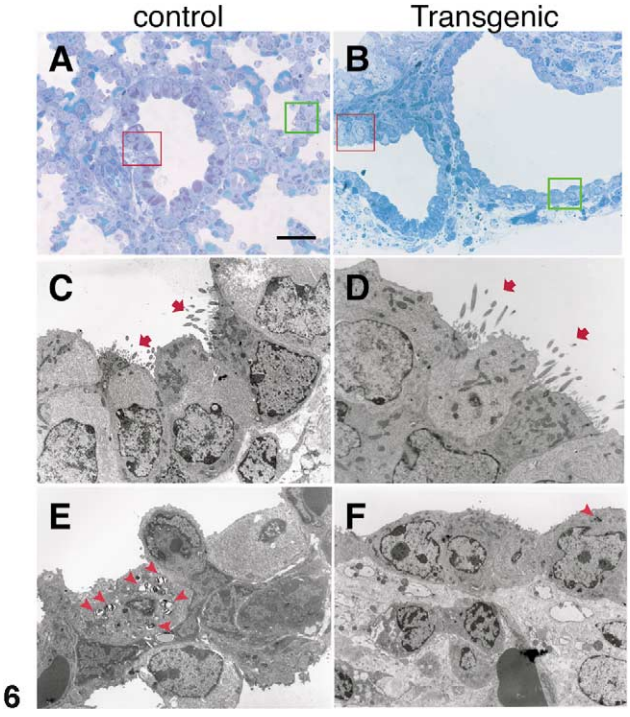


Fig. 5. Histological examination of E18.5 control and transgenic lungs. Sections of control (A, C) and transgenic (B, D) lungs were stained with heamatoxylin and eosin. (A, B) Low magnification images, and (C, D) high magnification images of the distal part of the lung (green squares in A and B). Scale bar, 200 μm for (A) and (B), 50 μm for (C) and (D).

saturated beads are implanted in the mesenchyme adjacent to proximal endoderm, where the genes are not normally transcribed. As shown in Fig. 3C and D, FGF7 very effectively induces ectopic *Pea*3, and to a lesser extent, *Erm* expression in the proximal endoderm, while control beads soaked with BSA have no effect (Fig. 3A and B). A much weaker response is seen with FGF10 (Fig. 3E and F). By contrast, FGF9 weakly induces ectopic *Erm* and *Pea3* expression in proximal epithelium, but gives strong upregulation of *Pea3* in the mesenchyme adjacent to the bead. As expected, given its broader specificity for receptors, FGF2 induces ectopic *Pea3* and *Erm* in both the endoderm and mesoderm, with the strongest effect on *Pea3* (Fig. 3G–J). Taken together, these results show that the two genes are downstream of FGF signaling in the lung and respond differentially to specific FGFs both in culture and in the intact lung.

Abnormal development of SpC-EngR-Erm transgenic lung

The overlapping expression patterns of *Erm* and *Pea3* described above suggest that they have redundant roles in lung development. This idea is supported by the fact that *Pea3*−/− mice survive postnatally and show no overt difference in growth rate and state of health, which would not be expected if they had lung abnormalities (Laing et al., 2000). It is also possible that members of other, more divergent, ETS protein subfamilies expressed in the embryonic lung (Elf, Ehf) (Ng et al., 2002) compensate for loss of Pea3 members. We therefore decided to take a transgenic approach to study the function of Erm in embryonic lung, using the well-characterized 3.7-kb human *SpC* promoter/enhancer (Wert et al., 1993). This drives transgene expression in the distal epithelium, but only at high levels from E15.5. We first expressed either full-length Erm protein or a truncated protein containing the ETS DNA binding domain, but lacking both the N-terminal activation domain and a small portion of the C terminus, which is considered to have slight activity for transcriptional activation. At E18.5, no abnormalities are observed in the lungs of embryos transgenic for either of these expression constructs. In the case of the full-length protein, these results may be because the expression level of the transgene is not high enough or may reflect the fact that the level of endogenous Erm is already saturating. For the truncated protein, it is likely that a very high level of expression is needed to affect cell behavior. This idea is supported by our in vitro studies, showing that the deletion mutant blocks less than 20% of Erm-mediated activation of a Pea3-artificial promotor-luciferase reporter (Fig. 4B). These in vitro studies also confirmed that, in an epithelial cell line derived from adult mouse lung, Erm acts as an activator of a reporter construct (Fig. 4C).

We then made transgenic embryos expressing a protein consisting of truncated Erm fused with the repressor domain of *Drosophila* Engrailed (EngR) (Fig. 4A). Our in vitro studies demonstrated that EngR-Erm is about five times more effective at blocking Erm induction of the luciferase reporter than the deletion mutant (Fig. 4B). A similar approach has been used successfully to generate repressor forms of ETS proteins and to study the function of ETS protein during sea urchin development (Wei et al., 1999), mammary gland tumorigenesis (Shepherd et al., 2001), and neural crest stem cell differentiation (Paratore et al., 2002). Six of a total of nine transgenic lungs at E18.5 showed an abnormal gross phenotype, being smaller than nontransgenics and paler, but with a normal lobular morphology (Fig. 4D and E). The expression of the transgene is confirmed by section in situ hybridization using the *SV40* small T intron as the probe (Fig. 4F and G). Higher levels of transgene expression correlate with a more severe phenotype.

Histological analysis at E18.5 reveals several significant differences between transgenic and normal lungs. First, the control lungs contain many small saccules (primitive alveoli) separated by a thin layer of mesenchyme. The epithelial cells lining the saccules are characteristic of squamous Type I cells interspersed by differentiated Type II cells. By contrast, transgenic lungs have clearly undergone less extensive branching of the epithelium and contain fewer, larger sacs separated by abundant mesenchyme and lined with cuboidal epithelial cells resembling epithelial precursors or immature Type II cells. In addition, the proximal epithelium extends closer to the pleural surface of the lung than normal, and the transition from proximal to cuboidal cells is abrupt. No difference is observed in the morphology of the convoluted epithelium of the proximal airways in transgenic and wild type lungs (Fig. 5).

To examine the ultrastructural morphology of the epithelial cells, transmission electron microscopy (EM) was performed at E18.5. This confirmed that control distal epithelium at this stage is lined with squamous Type I cells, interspersed by mature Type II cells. In contrast, the distal epithelium of the transgenic lungs is composed solely of cuboidal cells resembling immature Type II cells, some cells contain a few atypical lamellar bodies compared with mature cells. The morphology of the proximal epithelial

Fig. 6. Ultrastructural morphology of control and transgenic lung epithelial cells. (A, B) Ethylene blue-stained 500 nm sections of distal regions of control (A) and transgenic (B) lungs. (C–F) Transmission electron microscopy images from these sections. Proximal cells taken approximately within the red squares are shown for nontransgenic (C) and transgenic (D) lungs. Note the presence of ciliated cells (arrows). (E, F) From distal regions taken approximately within the green squares in (A) and (B). (E) shows a mature type II cell in the control lung epithelium characterized by lamellar bodies (red arrowhead). The distal epithelium of transgenic lung is composed of cuboidal cells resembling immature type II cells (F). Only a few atypical lamellar bodies were seen (red arrow). Scale bar, 25 μm.

cells of the transgenic lungs is not significantly different from wild type and both ciliated cells and nonciliated cells were present (Fig. 6). Together, these results suggest that expression of *SpC-EngR-Erm* result in the specific disruption of the differentiation of mature alveolar Type I and II cells.

Expression of the proximal markers, CC10 and Foxj1, in EngR-Erm transgenic lungs

To analyze epithelial differentiation at the molecular level, we examined expression of two markers for proximal endoderm, *Foxj1* and *CC10*, *Foxj1* encodes a winged helix transcription factor expressed from about E15 in the ciliated cells of the proximal airway, but not in distal epithelium (Hackett et al., 1995). CC10 is a secreted glycoprotein characteristic of nonciliated Clara cells of the proximal epithelium and is expressed from E17.5 (Wert et al., 1993). Both *Foxj1* and *CC10* are expressed in transgenic lungs, apparently at higher levels than normal (Fig. 7). The presence of CC10 protein in transgenic lungs is confirmed by CC10 antibody staining (Fig. 8A and B). Moreover, the relative proportion of cells that express the proximal marker, *Foxj1,* is higher in transgenic lungs, compared with controls (see Materials and methods). In situ hybridization of adjacent sections of a transgenic lung reveals that neither transgene nor *SpC* are transcribed in the epithelial cells expressing *Foxj1* (Fig. 7).

Abnormal differentiation of mature distal epithelial cells in transgenic lungs

Early in lung development, the epithelium is composed of a morphologically uniform population of precursor cells that express *SpC*. After E17.5, these give rise distally to flattened Type I alveolar cells that are specialized for gas exchange and characteristically express Aquaporin 5 (Aqp5), a water channel protein. These flattened cells are interspersed with rounded Type II cells, specialized for surfactant protein synthesis and expressing very high levels of *SpC*. In transgenic lungs, the layer of relatively uniform cuboidal cells that lines the dilated distal sacs and expresses the transgene expresses *SpC* (Fig. 7). However, no significant expression of *Aqp5* is seen, compared with control lungs (Fig. 8C and D). Taken together with the EM morphology, these results suggest that the population of distal cells in transgenic lungs at E18.5 consists of relatively undifferentiated precursor cells, and that EngR-Erm delays or inhibits their differentiation into mature Type I and Type II cells.

Cell proliferation in transgenic lungs

We estimated the mitotic index of cells of transgenic and control lungs by phosphohistone H3 antibody staining at E18.5 (Fig. 8E and F). The results indicate that the transgenic distal epithelial cells have an approximately sixfold higher proliferation rate than controls at this time. The mitotic index of the proximal epithelial cells is also increased by about ninefold in transgenic lungs at E18.5, compared with wild type. The mesenchymal cells appear to have elevated proliferation as well, although this is not quantitated. No difference in apoptosis between wild type and transgenic lungs is seen by immunohistochemistry with antibody to cleaved caspase-3 protein (data not shown).

Discussion

Pea3 sub-family members are new players in the epithelial–mesenchymal interactions at the distal tip of lung buds

Although *Pea3* family members are known to be expressed at sites of mesenchymal–epithelial interactions in several organs, including salivary gland, gut, lung, inner ear, and the olfactory organ (Chotteau-Lelievre et al., 1997), their roles in morphogenesis have not been investigated previously. In this paper, we show that *Erm* and *Pea3* have overlapping expression in the distal endoderm of the developing lung from as early as E10.5 after the buds have first appeared, while *Pea3* (like *Er81*) is also expressed in the mesoderm. (Chotteau-Lelievre et al., 1997; de Launoit et al., 1997). Moreover, we demonstrate that transgenic expression of a repressor form of Erm (EngR-Erm) results in a severe disruption of lung development. In addition to defects in branching morphogenesis, the differentiation of distal cell types is inhibited or delayed. Consequently, by E18.5, the distended terminal airways are lined almost entirely by cuboidal cells that express *SpC* but lack normal lamellar bodies, and squamous Type I cells transcribing *Aquaporin 5* are absent. However, the differentiation of proximal cell types expressing *CC10* and *Foxj1* does occur. Indeed, there is an increase in the relative proportion of proximal endodermal cells compared with distal.

One model to explain the transgenic phenotype is illustrated in Fig. 9. Based on the expression pattern of *Erm/Pea3* and on lineage studies in vivo (Perl et al., 2002), we propose that Erm/Pea3 act within a population of precursor cells at the tip of lung buds. These cells will give rise to mature Type I and Type II cells, and also have the potential to differentiate into proximal cell types. Erm/Pea3 normally functions cell autonomously as part of the program maintaining the distal precursor phenotype, and later to promote the differentiation of distal precursor cells into mature Type I and Type II cells. In transgenic lungs, *SpC-EngR-Erm* reduces the size of the precursor cell population and inhibits or delays the differentiation of mature distal cells, but still allows the precursor cells to differentiate into proximal cell types. Consequently, by E18.5, there is an increase in the relative proportion of proximal versus distal cells in transgenic lungs. The idea that EngR-Erm has a differential

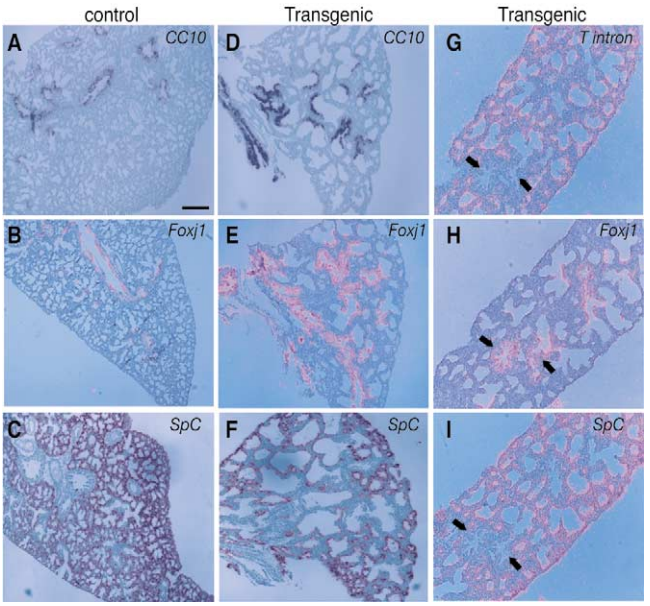


Fig. 7. Gene expression in E18.5 control and transgenic lungs. Radioactive section in situ hybridization was performed by using probes for (A, D) *CC10;* (B, E, H) *Foxj1;* (C, F, I) *SpC;* and (G) *SV40 T intron*. (A–C) control lung, (D–I) transgenic lungs. (D–F) and (G–I) are from two independent transgenic lines and (G–I) are adjacent sections. (A–C) were processed at the same time as (D–F). (A–F) In transgenic lungs, the expression levels of *CC10* and *Foxj1* are significantly higher than in wild type. Cells expressing *SpC* are present in large, distended sacs in the transgenic lungs but in numerous primitive alveoli in wild type. Arrows in (G–I) indicate the same regions of proximal endoderm that express *Foxj1* but not *SpC* or *small T intron*. By contrast, the latter two genes are coexpressed in the epithelial cells associated with the distended sacs. Scale bar, 100 μm.

effect on the differentiation of distal versus proximal lineages is consistent with recent studies on the role of Erm in neural crest stem cells (NCSCs) (Paratore et al., 2002). These authors show that the dominant repressor form of Erm blocks the differentiation of multipotent NCSCs into neurons, but not into glia cells.

One paradox of our results is that we observe high epithelial proliferation rates at E18.5 in transgenic lungs, even though the overall size of the lung is smaller than normal. One possibility is that Pea3/Erm normally help to maintain the higher proliferation rate of distal versus proximal epithelial cells during the pseudoglandular stage, but later, wild-type cells differentiate and their proliferation rate is reduced. In transgenic lungs, the population of proliferating precursor cells is smaller than normal but they still maintain a relatively high rate of proliferation up to E18.5.

Based on some studies showing that *Pea3/Erm* regulate the expression of matrix metalloproteinases (de Launoit et al., 2000; Crawford et al., 2001), it is also possible that Pea3/Erm regulate some of the dynamic cell–cell and cell–matrix interactions that are crucial for normal branching morphogenesis. For example, This might account for the fact that, when Erm/Pea3 action is blocked, there is less branching and large, dilated distal sacs are formed. The disruption of Type I epithelial cell differentiation and *Aquaporin 5* expression might then be secondary to the disrupted distal morphogenesis. However, this mechanism alone does not account for the specific phenotype that we see compared with other transgenic lungs in which branching morphogenesis is disrupted as a result of ectopic transgene expression. For example, in *SpC-TGF-β* (Zhou et al., 1996) *SpC-HNF-3β* (Zhou et al., 1997) transgenic lungs, and the lungs in which a transgene encoding a Gata6-Engrailed repressor fusion protein is overexpressed with the *SpC* promoter (Yang et al., 2002), the expression of both proximal and distal markers are reduced. In these examples, the distal epithelial cells may be blocked at an earlier developmental stage than the *SpC-EngR-Erm* transgenic lungs, and differentiation of both proximal and distal cell types is impaired.

We cannot rule out the possibility that the EngR-Erm transgene negatively regulates its own expression, since FGF7 upregulates *SpC* expression in isolated endoderm (Fig. 2E). However, if this is the case, then the transgenic phenotype that we observe is milder than might be expected for high expression of a dominant repressor protein.

Specificity of EngR-Erm action

The engrailed domain has been used frequently to block the function of various families of transcription factors, including ETS domain proteins (Wei et al., 1999; Novitch et al., 2001; Muhr et al., 2001; Yang et al., 2002). Maintenance of the functional specificity of DNA binding domains after fusion with the engrailed repressor domain has been shown for the bHLH proteins, Olig1 and Olig2 (Novitch et al., 2001). Expression of the Engrailed domain by itself does not cause lung developmental defects (Yang et al., 2002). However, our results do not rule out the possibility that, by expressing a repressor form of Erm, targets of some other ETS proteins, that recognize identical *cis* regulatory sequences, are also repressed. The most likely candidates are the targets of *Pea3*, whose expression overlap with *Erm* in the epithelium, although in a more restricted domain. In support of this idea, studies in mammary cell lines have shown that a Pea3-EngR fusion protein inhibits activation of a reporter by both Erm and Er81. Importantly, the Pea3-EngR fusion protein did not recognize the binding site for another ETS protein, GABP-α, in the MMTV promotor, even when expressed at high levels (Shepherd et al., 2001). Other ETS factors present in embryonic mouse lung epithelium are Ets2, Elf3, and the closely related Ehf (Ng et al., 2002), but based on molecular phylogeny, these proteins are more distantly related to Erm/Pea3 than GABP-α (Laudet et al., 1999). For these reasons, we consider the ETS proteins most likely to be blocked by EngR-Erm in our study are Erm and Pea3. In addition, any ETS protein that EngR-Erm does block is also likely to be one that has the ability to compensate for Erm/Pea3 loss.

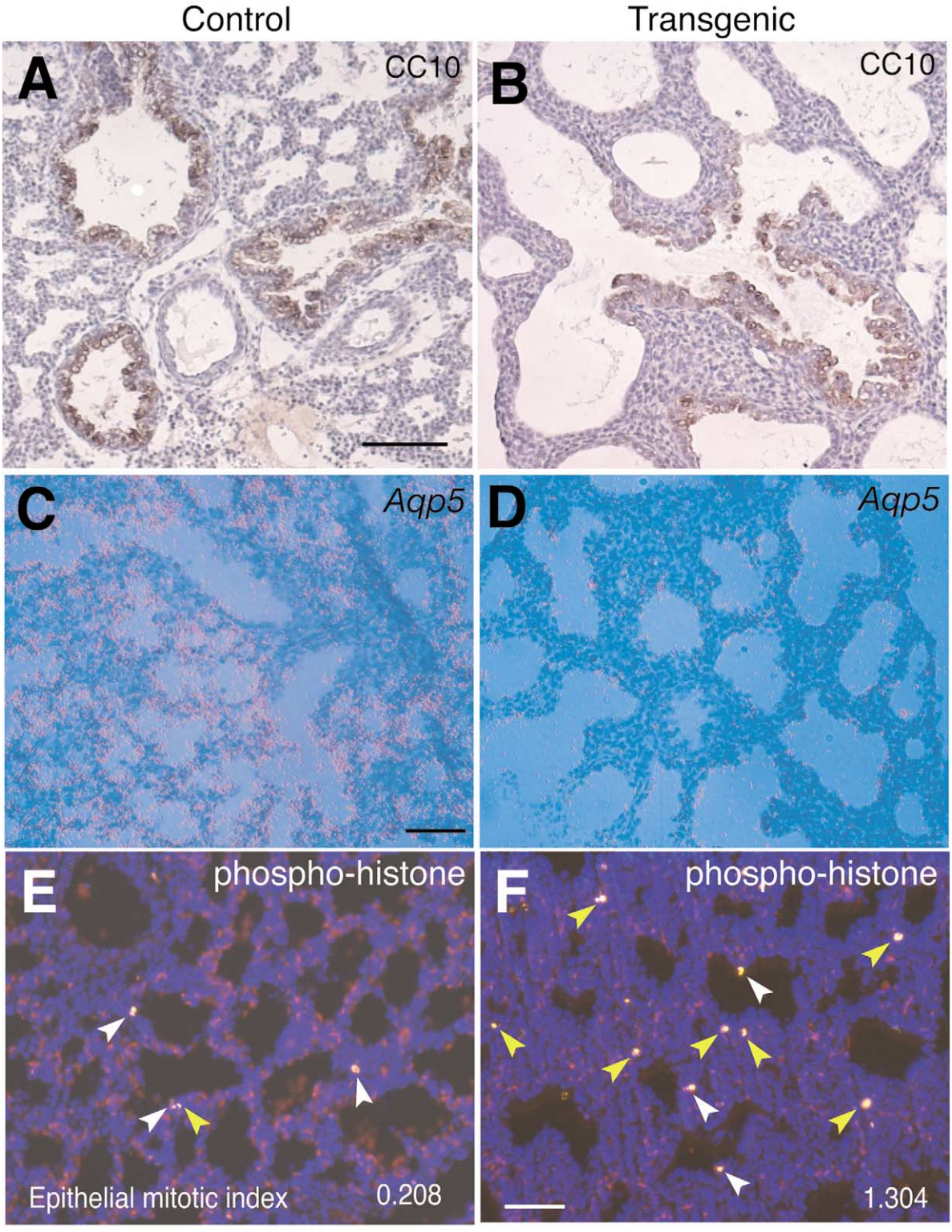


Fig. 8. Differentiation and proliferation of proximal and distal epithelial cells in transgenic lungs. (A, B) CC10 antibody staining of control (A) and transgenic (B) lungs. (C, D) Section in situ hybridization of control (C) and transgenic (D) lungs using *Aquaporin 5* as probe. (E, F) Proliferation of epithelial cells in control and transgenic lungs estimated by double labeling with anti phosphahistone H3 antibody and DAPI of control (E) and transgenic lungs (F). Yellow arrowheads indicate phosphohistone positive mesenchymal cells; white arrowheads indicate phosphohistone positive cells that line sacs, and are presumably epithelial cells. Nuclear staining is shown as blue. Autofluorescence of red blood cells shows as a characteristic red staining. Scale bar, 100 μm.

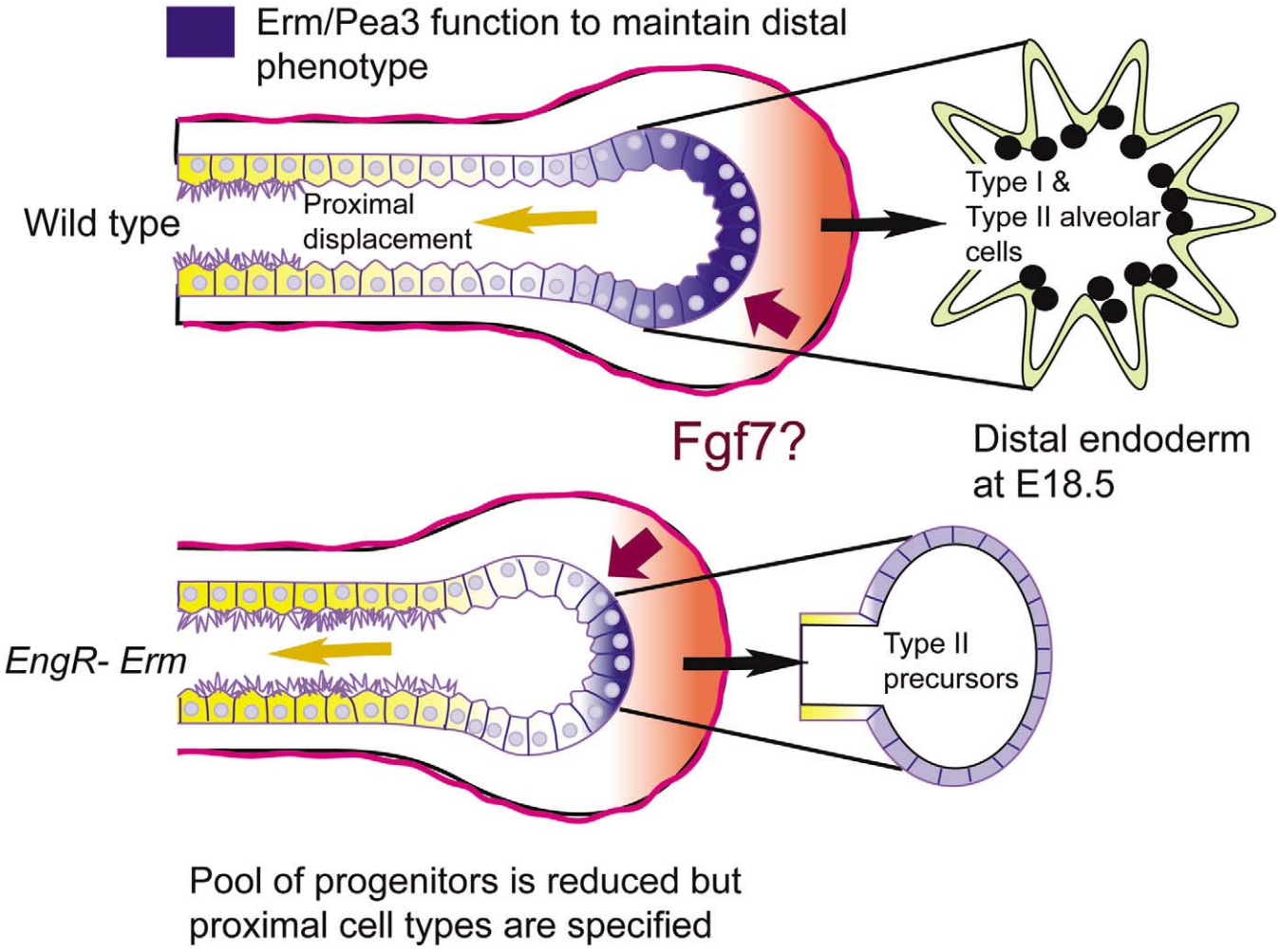


Fig. 9. A model for the function of Pea3/Erm in distal epithelium and the phenotype of *Spc-EngR-Erm* transgenic lungs. Pea3/Erm, possibly responding to Fgf7 in mesoderm (brown arrow), act to maintain the phenotype of the distal endodermal progenitor cells population (blue). As lung bud outgrowth continues, some progeny of these cells are displaced proximally (yellow arrow) and differentiate into proximal cell types (yellow). Shortly before birth, the precursor pool gives rise (black arrow) to mature Type I and Type II cells (green and black) that will contribute to the future alveoli. In *SpC-EngR-Erm* transgenic lungs, maintenance of the precursor phenotype is inhibited, but not differentiation of the distal into proximal cells. The reduced overall size of the precursor pool, coupled with inhibition of differentiation of mature Type I and II cells, blocks the development of future alveoli. As a result, the distal part of the transgenic lung is composed of relatively undifferentiated cells and the proportion of the proximal cells is higher than wild type.

All *Pea3* sub-family members have been genetically inactivated in mice. *Erm* null mutant dies at E9.5, before lung development starts (T. Jessel, personal communication). *Pea3*−/− male mice are infertile, probably because of neuronal dysfunction, but apparently do not have a lung phenotype (Laing et al., 2000), suggesting that *Pea3* and *Erm* have redundant functions during lung development. In addition, *Ets2*−/− mice have been generated by chimera rescue and show no overt phenotype in organogenesis (Yamamoto et al., 1998), and the *Elf3*−/− mouse also show no lung abnormalities (Ng et al., 2002). Our approach of using a repressor protein overcomes the problems of potential functional redundancy and early lethality with Pea3 family members and has allowed us to uncover for the first time a role for ETS proteins in lung branching morphogenesis.

Regulation of Erm and Pea3 by FGFs during lung development

Given the fact that Pea3 family members are downstream of FGF signaling in other tissues, we explored the possibility that expression of these genes in distal lung endoderm is maintained by FGF signaling from the mesoderm. Our studies support this hypothesis, but also show that expression in the epithelium responds differently to FGF7 and FGF10, even though both ligands can bind to FGF receptor 2b, an isoform that is expressed in the endoderm and is absolutely required for early lung development (Ornitz and Itoh, 2001 for review; Ornitz et al., 1996; Igarashi et al., 1998; De Moerlooze et al., 2000). This differential response was seen in both isolated endoderm and in intact lungs in vitro. In isolated endoderm, *Pea3* and, to a lesser extent, *Erm*, are

strongly upregulated in response to FGF7, which also promotes the expression of the distal markers, *SpC* and *Bmp4,* and the formation of cysts with numerous small buds. By contrast, FGF10 promotes the formation of elongated buds, and is much less effective than FGF7 in upregulating expression of *Bmp4*, *Pea3,* and *Erm*. The fact that differential effects are seen both in isolated endoderm and in intact embryonic lungs suggests that the results are not an artifact of culturing endoderm in the presence of Matrigel components, such as heparan sulfate proteoglycans and other extracellular matrix components that may alter the binding of FGFs to specific receptors (Igarashi et al., 1998).

The different effects of FGF7 and FGF10 on morphology and *SpC* and/or *Bmp4* expression have been observed previously with tracheal endoderm (Hyatt et al., 2002) but have not been reported before for genes encoding transcription factors. Taken together, the results suggest that FGF10 is mainly responsible for the initiation and outgrowth of epithelial buds, while FGF7 is mainly responsible for the maintenance of the expression of distal-specific genes and differentiation of distal cell types (Ulich et al., 1994; Simonet et al., 1995; Shiratori et al., 1996; Bellusci et al., 1997; Cardoso et al., 1997; Park et al., 1998; Shannon et al., 1999; Sekine et al., 1999; Tichelaar et al., 2000; Weaver et al., 2000). Since FGF7 is more effective than FGF10 for the induction of *Pea3/Erm* expression, the phenotype that we observed in our transgenic lung may result from a partial block of FGF7 signaling, while FGF10 signaling remains largely intact. In other words, Pea3/Erm may preferentially mediate signaling events between mesenchyme and epithelium that involve FGF7.

Potential target genes for Erm and Pea3 in the embryonic lung

Our results suggest that Erm and/or Pea3 regulate the expression of two classes of downstream targets. During the pseudoglandular stage, they may positively regulate products required to maintain a high rate of cell proliferation and an undifferentiated, multipotent phenotype in a population of distal progenitor cells. Later in development, they may promote the expression of genes driving the differentiation of mature Type II and Type I alveolar epithelial cells and/or the expression of products characteristic of these mature cell types. Recent studies have identified an important role for the matrix metalloproteinase, MMP7 or matrilysin, in epithelial cells in adult lung (Li et al., 2002). Studies in mammary epithelial cell lines have shown that Erm promotes the expression of MMP in response to Wnt signaling (Crawford et al., 2001). This raises the possibility that Erm also regulates metalloproteinase expression in the embryonic and adult lung in vivo. However, analysis of transgenic *EngR-Erm* lungs by in situ hybridization showed no inhibition in the level of *MMP14* transcripts, which is known to be expressed in lung epithelium at E18.5 (Kheradmand et al., 2001). Further analysis of gene expression in wild type and transgenic lungs will therefore be necessary to identify potential downstream targets of the Pea3 family in vivo.

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